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Certain substratum inhomogeneities; e.g., the presence of welds, influence early colonization events. At some point in time, however, biofilm biomass constituents, metabolic activity, and community structure become independent of substratum effects. Mixed species biofilms show CONTINUED

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A number of new on-line techniques were developed for dissecting bacterial adhesion and activity events at the cellular and molecular levels. A quartz crystal microbalance (QCM) gravimetric technique detected between 10^4 and 10^7 cells cm^{-2}. Both quantitative information concerning cells numbers and qualitative information concerning biofilm constituents was obtained via a Fourier transform infrared spectroscopy (FTIR) technique. Bioluminescence of lux strains of environmental bacteria was used as an endpoint for adhesion. Bacterial monocultures and consortial biofilms were shown to create electrochemical discontinuities on SS surfaces. These changes in open circuit potential (OCP) were shown to be transient. OCP measurements were employed as a measure of early fouling events. Electrochemical impedance spectroscopy, small amplitude cyclic voltommetry, and a scanning vibrating electrode technique were employed to demonstrate the influence of bacterial biofilms on corrosion activities.

Several new analytical biochemical methods were developed for dissecting microbial community structure in biofilms and in bulk-phase cultures. Improvements in analytical techniques enabled detection and characterization of eubacterial and archaebacterial components from extreme environments. Biofilm and planktonic populations were characterized at the cellular level (10^{-15} molar) using improved lipid extraction procedures, including supercritical fluid extraction/ chromatography.

Expression of genes associated with bacterial alginate production increases for P. aeruginosa when cells colonize SS substrata. The presence of several gene sequences associated with bacterial alginate production may be correlated with the adhesion event. Bacterial alginate production, as inferred from DNA homology studies, was associated with a majority of bacteria isolated from corroding pipeline surfaces in freshwater TVA pipelines.

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JOINT PROGRAM ON MOLECULAR BIOLOGY OF MARINE ORGANISMS

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I. ABSTRACT

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Certain substratum inhomogeneities; e.g., the presence of welds, influence early colonization events. At some point in time, however, biofilm biomass constituents, metabolic activity, and community structure become independent of substratum effects. Mixed species biofilms show evidence of

stress on 316 SS surfaces. The gross structure of these cells is not affected by the presence of certain metallurgical inhomogeneities.

Fluid shear stress was been shown to exert a significant effect on Alteromonas atlantic biofilms associated with stainless steel. At shear forces in the range of 3-10 dynes cm^{-2}, attachment increased with shear; decreases in unit cell metabolic activity were observed, however. Methods were developed for assessing the effects of environmental and cultural variables on biofilm development parameters under well-defined hydraulic conditions.

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II. OVERVIEW

Introduction. Bacteria possess a number of adaptive mechanisms for responding to those physicochemical factors which define their environment. These factors include nutrient availability, pH, Eh, temperature, organic and ionic content, and the presence of antagonistic agents. Depending upon the types and numbers present, bacteria can effect alterations in their physiology or physical state in response to the environment. Organic and inorganic acid production, heavy metal binding, transformation of xenobiotics, and extracellular polysaccharide production are important adaptive tools for bacteria in this regard. In most ecosystems, these activities are dependent upon the ability of bacteria to attach to surfaces.

That bacteria are a form of self-replicating organic and ionic particulate material distinguishes them from other, abiological, contaminants. In natural aquatic systems, the majority of bacteria are attached to surfaces. Indeed, surface area is a major limiting factor for microbial growth in nearly every freshwater and marine environment. The ratio of planktonic (free-floating) to sessile (attached) bacteria is a function of several interrelated factors. These include surface energetics, materials of construction, topology, hydraulic factors, and biofilm chemistry. It is the biofilm

which gives rise to biological particulates and by-products of metabolism which are responsible for biological fouling activities.

The impact of these microenvironmental alterations on various surfaces and fluid handling systems of industrial importance can be significant. Biological fouling can be defined in terms of its effects on various products and processes. Mechanical blockage of flowing systems, corrosion activities, product contamination, and impedance of heat transfer processes result from bacterial adhesion processes. The economic effects of these activities can be staggering.

Despite their omnipresence in most fluid process systems, relatively little is known about those factors which contribute to bacterial growth and replication in these dynamic environments. This overview and the conceptual model proposed herein address the role that attachment processes play in the survival, growth, and replication of bacteria in environmental and industrial systems.

Adaptive Advantages. There are several adaptive advantages which have been ascribed to a sessile existence. Zobell (1943) proposed that solid surfaces not only act to concentrate nutrients by adsorption, but also retard the diffusion of exoenzymes away from the cell--thus promoting the uptake of substrates which must be hydrolyzed extracellularly. Several workers have demonstrated that attachment processes

are, in part, a response to nutrient availability. Decreasing bulk-phase carbon-source concentrations in an aqueous system promote the attachment of marine (Marshall, 1988; Morita, 1982) and freshwater bacteria (Brown, et al., 1977). Geesey et al. (1978) showed that the predominant bacterial population in pristine mountain streams was associated with surfaces. Bacteria in industrial purified water systems, which have many similarities to natural oligotrophic ecosystems, also show a preference for surfaces (Mittelman et al., 1987).

Oligotrophic environments have been defined by some workers (Poindexter, 1981) as containing <1 mg L^{-1} total organic carbon. However, as Martin and MacLeod (1984) have observed, each bacterium and consortium possess a range of substrate and growth factor affinities. Whether growth in a particular environment will occur is a function of both the specific growth-promoting compounds as well as their relative concentrations. Attachment appears to be an important adaptive mechanism in what Morita (1982) has called the "starvation-survival" of bacteria in oligotrophic environments. The ability of sessile bacteria to elaborate a polyanionic, extracellular matrix may be an important factor in the concentration of trace nutrients from the bulk-phase environment (Marshall, 1986).

The ability of bacteria to form consortia in aqueous and terrestrial environments is essential for the survival of many species. Microorganisms isolated from natural environments

rarely exist as pure cultures. Rather, they live in diverse societies composed of individuals with various physiological and morphological characteristics. Animate and inanimate surfaces provide a matrix for the formation of these societies (Costerton et al., 1985). Many sulfate reducing, methanogenic, methylotrophic, and sulfur oxidizing bacteria are only found on surfaces in close association with microorganisms of diverse physiological activities which provide the narrow range of nutrients these microbes can use. For example, the dissimilatory sulfate-reducer, Desulfobacter sp., although frequently isolated from marine and brackish water environments, is difficult to recover as a monoculture. It is, however, easily grown in a consortium (Dowling and White, 1988). Substrate transfer is facilitated by the close association of organisms within these diverse populations (Mah et al., 1977).

The role of consortial members in the formation and activity of biofilms is an important piece of information missing from most adhesion studies. For example, classification of microorganisms involved at various stages of surface succession would be of value. That a differential sensitivity to oxidizing biocides exists within biofilm consortia on mild steel (Franklin et al., 1989) demonstrates the importance of using mixed cultures in adhesion studies.

Sessile bacteria are afforded a measure of protection from antagonistic agents present in bulk-phase aqueous

environments. Protection from lytic bacteria such as Bdellovibrio sps. (Venosa, 1975), the toxic effects of heavy metals (Mittelman and Geesey, 1985), and bactericidal agents (Costerton and Lashen, 1984; Costerton et al., 1981) are important advantages afforded to sessile organisms. This protective feature is a significant factor in many disease processes and biological fouling activities. Bacteria associated with biofilms are more resistant to antibiotic treatments which would otherwise prove effective against free-living populations (Hoyle et al., 1990). Apparent biocide resistance to potable water treatments is a serious problem throughout the world. LeChevallier et al. (1988) showed that attachment of Klebsiella pneumoniae to glass slides increased resistance to free chlorine disinfection by a factor of 150. While the high concentrations and multiple sites of action associated with many biocides may, for example, overwhelm plasmid-mediated resistance, some biofilm organisms may be resistant by virtue of their ability to form polyanionic extracellular polymeric substances (EPS). Unfortunately, most biocide efficacy studies employ planktonic bacterial populations as challenge organisms. Thus, treatments for biofouling problems very often are ineffective and, in some instances, can exacerbate existing fouling activities (Ruseska, et al., 1982).

Biofilm Development. Bacterial attachment and the subsequent formation of a biofilm appear to take place in a three stage process. In the first stage, surfaces are rapidly coated with an organic "conditioning" film. In the blood, this film might consist of proteinaceous compounds such as albumin (Absolom et al., 1987; Uyen et al., 1990); on teeth, mucopolysaccharides (Embery et al., 1986); in freshwater environments, humic substances (Marshall 1986). In studies of blood interactions with foreign substances, it was found that within 5 s most surfaces become uniformly coated with strongly adherent proteinaceous films (Baier and Dutton, 1969). Rapid modification of clean surfaces in marine waters by glycoprotein and polysaccharide compounds has also been reported (Baier, 1972; Corpe, 1970).

In the second stage of adhesion, single bacterial cells are transported to surfaces and reversible bonds are formed between the cell wall and surface. Bacterial EPS appear to mediate the attachment of primary colonizers to organic conditioning films associated with animate and inanimate surfaces (Marshall et al., 1971). Corpe (1970) described an "acid polysaccharide" which acts as a polymer bridge between bacterial cell walls and surfaces. It is interesting to note, however, that Allison and Sutherland (1987) have presented evidence that extracellular polysaccharide, while important in the development of surface films, is not directly involved in the initial adhesion process of freshwater bacteria. The high

uronic acid content of many types of EPS probably plays an important role in concentrating essential substrates and preventing antagonistic agents from reaching labile cellular processes. Calcium and other divalent cations may be involved in the initial stages of attachment. These ions appear to stabilize the EPS by cross-linking adjoining sugar groups within the polysaccharide polymer. Applegate and Bryers (1991) have described the role that calcium plays in stabilizing EPS to the effects of fluid shear.

The mature or third-stage biofilm consists of the organic conditioning film, a succession of colonizing bacterial consortia with their associated EPS, and various biofilm-associated detrital particles and ionic species. It is this structure which gives rise to the planktonic bacteria and their by-products (e.g., endotoxins) which act as self-replicating, particulate contaminants.

The question of whether differing substratum surface properties are communicated to the initial or succeeding organisms through the conditioning film is of great interest. Uyen et al. (1990) suggest that substratum properties can be transferred by an adsorbed protein film to adhering eucaryotic or procaryotic cells. They base this supposition on their finding that the amount and surface structure of albumin adsorbed onto inanimate surfaces was a function of substrate wettability (surface free energy). However, the possibility remains that bacteria recognize--and are attracted to--

moieties within the conditioning film rather than structures on the underlying surface. Despite recognition of the importance of conditioning films as precursors to biological fouling activities, treatments have not been developed for their control or modification. If, for example, the constituents of a conditioning film elicit a chemotactic response, the chemistry and topology of a given surface might exert a relatively minor influence on biofilm development. The type of conditioning film present may be the major factor involved with the penetration of pathogenic organisms into tissues, tooth surfaces, and implantable medical devices. It may also affect the rate and extent of such surface perturbations as pitting corrosion activities.

Adhesion Mechanisms. Mechanisms for bacterial surface recognition and initial colonization have yet to be fully elucidated. Some workers have described a chemotactic response by bacteria to surface-associated compounds. Chemotaxis is the movement of an organism in response to a chemical gradient. Young and Mitchell (1972) have implicated positive chemotaxis in the initial colonization of bacteria to marine surfaces such as ship hulls. The phenomenon of "negative chemotaxis" in response to surface-associated toxic substances has also been described (Chet et al., 1970; Young and Mitchell, 1973). Models of bacterial chemotaxis to surfaces in flowing aqueous systems have been described

(Keller and Segeal, 1971; Lepidus, 1980). Implicit in the chemotaxis mechanistic theory, however, is the ability of bacteria to direct their movement towards the surface. Since non-motile bacteria are also associated with fouling biofilms, chemotaxis can only be used to describe one factor involved in the initial attachment mechanism.

Impedance of flagellar activity by mechanical contact with surfaces appeared to promote attachment and lateral flagella formation in a marine vibrio (Belas and Colwell, 1982). Upon contact with a surface, the polar flagella of Vibrio parahemolyticus ceased to function. Shortly thereafter, lateral flagella formed around the cells, apparently mediating the "irreversible" attachment process.

Pilus mediated adhesion to gastrointestinal surfaces is recognized as a major factor in the toxicity of enteropathogenic Escherichia coli in humans and other animals (Gorbach et al., 1975). Pilus negative E. coli are incapable of attaching to these surfaces and producing pathogenic effects. In most cases, adhesion to host tissues by pathogenic bacteria is a first step in initiating infections (Savage, 1985). Usually, this process is mediated by carbohydrate-specific lectins on the bacterial cell surface (Jann and Hoschutzky, 1990). Ridgway and Olson (1981) presented scanning electron microscopic evidence which indicated that attachment of bacteria to potable water pipelines was mediated by extracellular fibrillar appendages.

Finally, and perhaps most significantly, physical characteristics of bacteria and substrata such as surface energy (Absclom et al., 1983; Baier et al., 1968; Fletcher, 1988, van Dijk, et al., 1988; van Loosdrecht and Zehnder, 1990), hydrophobic effects (Dahlback et al., 1981; van Loosdrecht et al., 1990; Rosenberg and Kjelleberg, 1986), and surface topology (Characklis, 1973b; McCoy et al., 1981; Corpe, 1980) may influence the initial attachment process. The DLVO theory of colloid stabilization, as applied by Marshall, et al. (1971) to bacterial colloids, holds that the separation between bacteria and adsorbents in an electrolyte solution is dependent upon a balance between attractive and repulsive forces. This relationship can be described by the following equation:

$$V_t=V_r+V_a \quad (1)$$

where, V_t=total energy of interaction

V_r=energy of repulsion

V_a=energy of attraction

The electrical double-layer, which is associated with surfaces in electrolyte or dilute organic solutions, is thought to present a repulsive barrier to bacterial-adsorbent interactions. This repulsion, however, is balanced by the short-range van der Waals forces which attract colloids to

surfaces in such environments. The separation distance is minimized under conditions of low surface potential and bacterial affinity for surfaces is maximized at increased salt concentrations. However, above a salt concentration of 0.1 M, Fletcher (1988) found that bacterial attachment began to decline exponentially with increasing cation concentration. Firm ("irreversible") attachment requires some mechanism whereby the finite separation distance imposed by the electrical double-layer is bridged. Corpe (1980) and others believe that EPS are involved in this bridging process.

There is also evidence that hydrophobic interactions play a role in controlling adhesion processes. As bacteria approach a surface, this theory holds that water molecules are displaced, creating a lower free energy of interaction. When two surfaces covered by ordered layers of water molecules approach one another, layers of water are released into the bulk-phase from between the two surfaces. This results in a decrease in free energy and an increase in entropy. Depending upon the degree of interaction between the cell and a surface, this process can favor adhesion (Rosenberg and Kjelleberg, 1986). Surface energetics tends to favor colonization of bacteria to low energy (hydrophobic) surfaces such as Teflon. In general, colonization, but not necessarily irreversible adhesion, is greatest for bacteria possessing hydrophobic cell surfaces interacting with hydrophobic substrata.

The data are often confusing, however, with respect to the mechanisms of bacterial adhesion processes. While surface energy considerations would appear to favor adhesion of bacteria to low energy (hydrophobic) surfaces such as Teflon (Fletcher and Loeb, 1979), Absolom et al. (1983) pointed out that preferential adhesion to relatively high energy (hydrophilic) surfaces can occur when the surface energy of the bacteria is greater than that of the suspending medium. Clearly, the surface energies of substrata (and their associated conditioning films), bacterial cell surfaces, and bulk-phases are interdependent variables.

Environmental Factors Influencing Adhesion Processes. Studies of environmental and substrata variables which influence the initial attachment of bacteria and the subsequent formation of mature biofilms have generally lacked relevance to the actual in situ conditions. Previous studies by Fletcher et al. (1976,1979,1988), Dexter et al. (1975), Marshall et al.(1971), Rosenberg et al. (1980,1981), and others have focused on physicochemical interactions between bacterial monocultures and glass or polystyrene surfaces. The effects of varying bulk-phase conditions, surface characteristics, in situ conditioning films, and other environmental factors on attachment processes have not been fully explored. Preliminary work by Corpe (1970), Dexter et al. (1975), and Fletcher (1976) did indicate, for example, that organic

conditioning films have an effect on attachment processes. The nature of the conditioning films and their relative importance for initial attachment were not described, however.

Bacterial attachment typically occurs under the influence of some type of hydraulic influence. Characklis (1973a, 1973b) and McCoy and Costerton (1982) have demonstrated that such fluid hydraulic parameters as turbulence and fluid velocity have an effect on the nature of biofilms. Below a critical shear force, however, the amount of biomass per unit area does not appear to be significantly reduced at increased linear fluid velocities. Indeed, Mittelman et al. (1990) showed changes in the biomass of Alteromonas atlantica biofilms associated with stainless steel were positively correlated with shear up to a critical fluid shear.

Metabolic Influences on Adhesion. A key to the understanding of adhesion processes is the ability to elicit biofilm function from structure and activity. In studies of biomass formation and mineralization, sessile bacteria have usually demonstrated significantly higher activities than have planktonic populations. For example, a natural population of sessile marine bacteria was found to transport ATP 100 times faster than planktonic organisms on a per cell basis (Hodson et al., 1981). The nature of extracellular polysaccharides also differs between sessile and planktonic bacteria. Valeur et al. (1988) demonstrated that a significant difference

existed between the ratio of saturated: unsaturated fatty acids and poly-betahydroxybutyrate (PHB) content between planktonic and sessile bacteria. A subpopulation of the test organism containing a higher ratio of total C18/C16 fatty acids and lower PHB levels selectively adhered to test surfaces. Nichols et al. (1985) demonstrated that significant shifts in the IR spectra of P. atlantica biofilms as measured by Fourier transform infrared spectroscopy (FTIR) result when the organism is grown under different growth conditions. Environmental factors such as bulk-phase C:N ratio and fluid shear also appear to affect the nature and activity of biofilms on surfaces such as stainless steel.

Bartlett et al. (1988) have shown that extracellular polysaccharide production by P. atlantica is a variable trait, determined by expression of a now defined "eps" locus. Mucoid colonies (eps^+) produce extracellular polysaccharide while the spontaneously reverted crenated colonies (eps^-) apparently lack the ability to synthesize large amounts of extracellular polysaccharide. When recombinant plasmids with a locus for extracellular polysaccharide production are transferred to crenated variants, expression of the mucoid (eps^+) form results. What effect expression of this eps gene has on attachment characteristics is as yet undetermined. A clearer picture of extracellular polysaccharide function in the development of primary and mature biofilms could lead to a better understanding of those factors which control the

transition of bacteria from a planktonic to a sessile existence. An alteration of extracellular polysaccharide composition or quantity in response to some environmental perturbation could be an important factor in this transition process.

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III. WORK ACCOMPLISHED

Biomass from Five-Day Biofilms is Homogeneously Distributed.

Reproducible colonization of replica stainless steel (SS) substrata in a laminar flow environment has been demonstrated. Surfaces can be uniformly colonized by axenic and mixed cultures of aerobic, facultatively anaerobic, and anaerobic bacteria isolated from freshwater environments. Substrata with five-day old "climax" populations exhibited a homogeneous biomass distribution (Mittelman et al., 1992b). Studies are currently underway to determine whether the rate of colonization is uniform across SS surfaces.

Bulk Phase Composition is a Determinant of Biofilm Structure.

Biofilm community structure is a function of bulk phase composition. The anaerobic, dissimilatory sulfate reducing bacterium, Desulfovibrio gigas, failed to colonize clean SS substrata in aerobic environments. However, SS substrata that were precolonized with the aerobic, slime-forming bacterium, Pseudomonas fluorescens, could support viable populations of D. gigas on SS in an aerobic system (Mittelman et al., 1992c). Greater numbers of the facultative anaerobe, Hafnia alvei, were associated with SS substrata that were previously colonized with P. fluorescens than were associated with clean SS.

Corrosion of 316 SS Substrata (Including Thin Films) is not Initiated by a Consortium Which Includes D. gigas.

In five-day adhesion experiments, a consortium composed of Pseudomonas fluorescens, D. gigas, Hafnia alvei, and Bacillus subtilis failed to initiate corrosion on welded and non-welded 316 SS substrata as measured by open circuit potential and electrochemical noise techniques. Neither preferential bacterial colonization nor differential activity were associated with metallurgical inhomogeneities (Mittelman et al., 1992c). In an independent experiment performed over a 41 day period, a consortium composed of P. aeruginosa, Citrobacter freundii, D. gigas, B. subtilis, and H. alvei failed to initiate corrosion on thin films of 316 SS as

measured by FTIR spectroscopy (G. Geesey, MSU, personal communication). Work is in progress to study differences in short-term colonization rates as a function of metallurgical inhomogeneities.

Characterization of Biofilm Biomass Constituents and Metabolic Activity

Community structure and metabolic activity have been resolved in several extreme environments using lipid biomarker techniques. New techniques involving less environmentally damaging solvents have been developed for the extraction and analysis of lipids from environmental samples (Hawthorne et al., 1992). Bacteria in five-day biofilms exhibited extensive, refractile intracellular granulation suggestive of poly-ß-hydroxy alkanoates (PHA). This evidence was supported by FTIR spectroscopy, which demonstrated the presence of polyester compounds in the biofilms. In addition, few of the cells in these biofilms were dividing (Mittelman et al., 1992c). Geesey and White (1990) reviewed bacterial growth and metabolic activity at solid-liquid interfaces.

Changes in cellular concentration and composition of a monoculture of Alteromonas atlantica were shown to be a function of the applied shear force (Mittelman et al., 1990). At shear forces in the range of 3-10 dynes cm^{-2}, attachment as measured by direct microscopic counts was greatest at the higher shear forces. ^{14}C-Acetate uptake activity on the

stainless steel surfaces ranged from 1 X 10^{-5} to 19 X 10^{-5} μmol cm^{-2} between 0.15 and 30 dynes cm^{-2} for 30 min uptake periods. On a per cell basis, however, activity decreased with shear, indicating a shift in metabolism. FTIR analyses revealed that protein and carbohydrate concentrations also increased with the applied shear. Increased biofilm C:N ratios and total fatty acids were associated with the higher shear stresses.

Low and White (1988) reviewed methods for studying two responses of specific microorganisms to metal substrata in the marine environment. The formation of hydrophobic protein fimbriae and the elaboration of acidic extracellular polymer polysaccharides as the biological manifestation of irreversible attachment were described. White et al. (1990a), White and Wilson (1989), and White (1988) reviewed the application of biochemical analyses to the study of environmental contamination by xenobiotics. Mittelman and White (1992) described the application of bacterial bioluminescence and fluorometry for assessing the efficacy of antifouling coatings for marine structures.

Development of Molecular Probes for the Characterization of Adhesion Processes.

Several freshwater biofilm isolates from corroding pipeline surfaces probed positive for various *alg* biosynthetic genes, providing preliminary evidence of a role for alginates in adhesion/corrosion processes (Wallace et al., 1992).

Bacterial alginate production, as inferred from DNA homology studies, was associated with a majority of bacteria isolated from corroding pipeline surfaces in freshwater TVA pipelines. Thirteen different organisms were identified by their fatty acid patterns as belonging to genera including Pseudomonas, Xanthomonas, Aeromonas, Bacillus, and Hafnia. Of these, 10 probed strongly with algD (GDP mannose dehydrogenase), 10 with algG (epimerase), 8 with alg76 (polymerase Rx), and 12 with algB (a regulatory gene). Six of the thirteen isolates probed strongly with all 4 genes. Work is underway to further explore the relationship between the presence of alg sequences and adhesive characteristics.

Molecular probes (DNA, 16s rRNA) have been utilized in studies of biofilm community structure and functional activity (Mittelman et al., 1992c; Wallace et al., 1992). A greater proportion of attached Pseudomonas aeruginosa cells exhibited expression of alg C promoter activity than did planktonic cells. Approximately 1/3 of the attached cells showed alg C activity. Studies in progress are examining the influence of substratum and bulk phase properties on the expression of genes which code for bacterial alginate production.

On-Line Analysis of Bacterial Biofilms.

Progress has been made in developing field-applicable, on-line monitoring tools for biofilm formation and accivity at inanimate surfaces (Nivens et al., 1991a; 1991b). Attenuated

total reflection Fourier transform infrared spectroscopy (ATR-FT/IR) was used to provide molecular details about the inner portion of biofilms by generating infrared absorption spectra from bacteria located within approximately 1 μm of the surface of germanium crystals (Nivens, et al., 1992a). The spectra produced information about functional groups of macromolecule such as amide linkages associated with proteins, ester bands of storage products such as poly-ß-alkonoates (PHA), and C-O stretches of extracellular polymer material consisting primarily polysaccharides. The technique has a detection limit of 5×10^5 Caulobacter crescentus cells/cm^2 (Nivens et al., 1992a). A three-channel spectrometer was designed to facilitate ATR-FT/IR studies by allowing experiments to be performed in parallel instead of sequential (Nivens et al., 1992b). Thus, the spectrometer can be used to save time and examine the effect of different treatments applied to biofilms developed under identical conditions.

The quartz crystal microbalance (QCM), utilizing a piezoelectric resonator, was also used to monitor the attachment and surface growth of C. crescentus (Nivens et al., 1991b) and Psuedomonas cepacia biofilms (Nivens et al., 1992c). Calibration curves of frequency shift versus biofilm cell counts were generated. The detection limits were found to be 5×10^4 and 3×10^5 cells/cm^2, respectively and the dynamic range of the technique was determined to be 2 orders of magnitude. To our knowledge, these studies were the first

to use a QCM for long-term (days), on-line monitoring in aqueous environments.

Experiments were completed on an on-line bioluminescence adhesion assay. Biofilm cell numbers and metabolic activity showed a significant positive correlation with light production by a lux construct of P. fluorescens (Mittelman et al., 1992a). Validation experiments demonstrating the utility of multipurpose, laminar-flow cells for in situ colonization and adhesion assays were completed (Mittelman et al. 1992b).

Changes in the open circuit potential of stainless steel surfaces were associated with the development of marine and freshwater biofilms (Mittelman et al., 1992b). M.W. Mittelman reported on molecular mechanisms of adhesion and on-line monitoring techniques for biofilm detection at Gordon Conferences during the summer of 1991.

Role of Biofilms in Microbially Influenced Corrosion Activity.

Corrosion potentials were mapped on the surfaces of mild steel as a function of microbially influenced corrosion (MIC) activity using scanning vibrating electrode technologies (Franklin et al., 1990; 1991a; 1991d; White et al., 1991a). This technique enabled studies of the spacial and temporal relationships between bacterial biofilms and anodic/cathodic reactions at metal surfaces. Reviews of microbially influenced corrosion mediated by mixed species biofilms were prepared (Dowling et al., 1991; Franklin and White, 1991).

Methods were developed for on-line electrochemical monitoring of biofilm effects on stainless steel via electrochemical impedance spectroscopy, small amplitude cyclic voltommetry, and OCP in freshwater and marine test systems (Dowling et al., 1989a; 1989b; Franklin et al., 1991e; White et al., 1990b; 1991b).

Franklin et al. (1991b; 1991c) described the effects of various environmental factors on biofilms and metallic substrata in aqueous environments. Bacterial biofilms were shown to cause pits, initiated by chemical corrosion, to continue to propagate either by reducing the efficacy of phosphate as a corrosion inhibitor or by maintaining the aggressive environment within pits. A test system was devised (Franklin et al., 1988) to assess the effects of chlorine and chlorine/bromine combinations (Franklin et al., 1991c) on biofilms associated with corroding surfaces. Treatments with 16 mg L^{-1} concentrations of halogen combinations, though effective at reducing bacterial numbers and activities, increased the corrosion rate of carbon steels.

Development of Sensitive Chemical Methods for Biofilm Analysis.

New methods were developed for rapid extraction and analysis of surface-associated microbial lipids in aqueous (Hawthorne et al., 1992) and sediment (Tunlid et al., 1991) environments.

An ultrasensitive method was developed for analyzing biomarker fatty acids from sessile and planktonic microorganisms using gas chromatography (GC) and mass spectrometry (Tunlid et al., 1989a). This method was utilized to examine the fatty acid membrane composition of free and attached Alteromonas atlantica cells growing on stainless steel coupons in a Fowler cell adhesion module. Notably, there were significant differences in the fatty acid composition of free and attached cells. The GC/MS method was also utilized to examine bacteria in biofilms attached to mild steel coupons (Dowling et al., 1988) and to characterize bacteria associated with cucumber roots (Tunlid et al., 1989b).

The molecular profile of fungal zoospores adhering to a solid surface was examined using Fourier transform infrared spectroscopy (FTIR) in the attenuated total reflectance (ATR) mode (Tunlid et al., 1991). The analyses demonstrated that the spores attached rapidly within a few minutes using proteinaceous substances as adhesins.

Techniques for assessing biomass constituents, metabolic activity, and physiological status in environments as diverse as anaerobic salt marsh concretions (Coleman et al., 1992) and methanogenic bioreactors were reported (Hedrick et al., 1991a; 1991b; 1992a). The effects of starvation and overfeeding regimes on community structure and activity were determined. The ratio of ^{14}C-Acetate incorporated into eubacterial and

eukaryotic fatty acids to methanogen ether lipids significantly increased with starvation and significantly decreased under overfeeding.

Guckert et al. (1991) described the application of lipid biomarker analyses to the dissection of methylotroph communities. PLFA phenotypic relationships compared favorably with phylogenetic associations based on 16s rRNA data for methylotrophs. Nichols and White (1989) showed that poly-ß-hydroxybutyrate accumulated in soil from a methane-enriched, halogenated hydrocarbon-degrading soil column. This work demonstrated the applicability of biochemical procedures to monitor populations of native soil microorganisms capable of degrading pollutants.

Two reviews concerning the application of biochemical analyses to the study of microbial consortia in environmental samples were prepared (Tunlid et al., 1990; 1992).

Chemical Analyses of Archaebacteria from Extreme Environments

A simplified version of the lipid extraction and derivatizations methods used in this laboratory was developed for distinguishing eubacterial from archaebacterial cultures by FTIR. The procedure depends upon differences in their membrane lipids: eubacteria contain ester-linked and archaebacteria contain ether-linked alkyl chains. The ratio of carbonyl (1743 cm^{-1}) to methyl (2924 cm^{-1}) absorption was found to be the most reliable way to distinguish pure cultures

(Hedrick et al., 1991d). Three species of eubacteria (Escherichia coli, Bacillus subtilis, and Micrococcus lysodeikticus) were clearly distinguished from 3 species of archaebacteria (Methanobacterium formicicum, Sulfolobus acidocaldarius, and Thermoplasma acidophilum).

Thermodesulfotobacterium commune, an unusual eubacterium containing both ester- and ether-linked membrane lipids, was chosen as the most difficult test of the method. It was distinguished from both the other eubacteria and the archaebacteria. The method as described was reliable on sample sizes from 1 mg to 30 mg dry weight of cells. The method was developed for distinguishing pure cultures, but it was not suitable for determining the ratio of archaebacteria to eubacteria in environmental samples.

A supercritical fluid chromatograph was constructed for the analysis of membrane ether lipids as biomarkers for the archaebacteria (Hedrick et al., 1991e). The standard lipid protocol used in this laboratory for the determination of bacterial and eukaryotic polar lipid fatty acids was modified to allow the simultaneous determination of archaebacterial ether lipids. While eubacteria have most of their membrane lipids as polar lipids, some archaebacteria were found with the majority of their membrane lipids in the glycolipid or the lipid-extracted residue fractions. This gave another dimension of information to the lipid profile. Many more isolates must be analyzed by this method before the extent and

phylogenic significance of archaebacterial ether lipid diversity can be determined

Data from a preliminary sampling of a deep-sea hydrothermal vent environment showed very high variability in microbial biomass and community structure (Hedrick et al., 1992b). Some samples showed very high levels of polyunsaturated fatty acids (up to 39.6% of polar lipid fatty acids), with the same polyunsaturates as have been found in barophilic bacteria. The origin of essential polyunsaturated fatty acids in barophilic bacteria has implications for marine nutrient webs, and is a possible source of essential fatty acids in human nutrition.

A horizon internal to the vent chimney material was found containing much more archaebacteria than eubacteria, and most of the eubacteria were probably thiobacilli (Hedrick et al., 1992b). Whether this represents a symbiotic or commensal relationship between these microbial groups, or whether they were just living in adjacent horizons of the chimney material could not be distinguished by this sampling. The potential of lipid biomarker analysis for determining the in situ biomass and community structure of the hydrothermal vent environment was demonstrated.

IV. RESEARCH QUESTIONS DEVELOPED AS PART OF THIS PROJECT: FUTURE RESEARCH DIRECTION

1. How does the secretion of exopolymeric substances by initial colonizing population(s) affect subsequent colonization by succeeding organisms?

2. How do bulk phase chemical and transport properties influence bacterial activities in the biofilm?

3. Does the secondary heterogeneity created by the primary colonizing population influence substratum stability?

4. Is bacterial alginate an important factor in the irreversible attachment of cells to inanimate substrata?

5. Can a lux construct be developed which acts as a reporter for bacterial alginate production?

6. Can colonization, metabolic activity, and exopolymer production be resolved at the level of individual bacterial cells?

7. Are there specific moieties associated with inanimate substrata which act to induce production of extracellular polymeric substances?

8. What are the chemical constituents of conditioning films which are associated with heavily fouled substrata?

9. Can a correlation be established between conditioning films and colonization/biofilm stability?

10. Can remedial measures be targeted to specific episodes in the colonization and/or adhesion event(s); e.g., exopolymeric substance formation?

11. What role do extracellular organelles such as flagella and fimbriae play in attachment to inanimate substrata?

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VI. PATENTS PENDING/FILED.

No patents were filed resulting from this research.

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